Occurrences of septuple and elevated Pfdhfr-Pfdhps quintuple mutations in general population threatens the use of sulfadoxine-pyrimethamine for malaria prevention during pregnancy in Eastern-coast of Tanzania

George M. Bwire (georgebwire@gmail.com)
Muhimbili University of Health and Allied Sciences School of Pharmacy
Wigilya P. Mikomangwa
Muhimbili University of Health and Allied Sciences School of Pharmacy
Manase Kilonzi
Muhimbili University of Health and Allied Sciences School of Pharmacy

Research article

Keywords: Pfdhfr, Pfdhps, Sulfadoxine-pyrimethamine, Septuple, Quintuple, Tanzania

Posted Date: April 8th, 2020

DOI: https://doi.org/10.21203/rs.2.20112/v2

Version of Record: A version of this preprint was published on July 22nd, 2020. See the published version at https://doi.org/10.1186/s12879-020-05253-7.

Abstract

Background: Plasmodium falciparum dihydrofolate reductase (Pfdhfr) and dihydropteroate synthetase (Pfdhps) mutations compromise the effectiveness of sulfadoxine-pyrimethamine (SP) for treatment of uncomplicated malaria, and are likely to impair the efficiency of intermittent preventive treatment during pregnancy (IPTp). This study was conducted to determine the level of Pfdhfr-Pfdhps mutations, more than one decade since SP was limited for IPTp use in pregnant women in Tanzania.

Methods: Parasite genomic DNA was extracted from dried blood spots prepared by finger prick. Extracted DNA were sequenced using a single MiSeq lane combining all PCR products. Genotyping of Pfdhfr and Pfdhps mutations were done using bcftools as well as custom scripts to filter and translate genotypes into drug resistance haplotypes.

Results: The Pfdhfr was analyzed from 445 samples, the wild type (WT) Pfdhfr haplotype NCSI was detected in 6 (1.3%) samples. Triple Pfdhfr IRNI (mutations are bolded and underlined) haplotype was dominant, contributing to 84% (number [n]=374) of haplotypes while 446 samples were studied for Pfdhps, WT for Pfdhps (SAKAA) was found in 6.7% (n=30) in samples. Double Pfdhps haplotype (SGEAA) accounted for 83% of all mutations at Pfdhps gene. Of 447 Pfdhfr-Pfdhps combined genotypes, only 0.9% (n=4) samples contained WT gene (SAKAA-NCSI). Quintuple (five) mutations, SGEAA-IRNI accounted for 71.4% (n=319) whereas 0.2% (n=1) had septuple (seven) mutations (AGKGS-IRNI). The overall prevalence of Pfdhfr K540E was 90.4% (n=396) while Pfdhps A581G was 1.1% (n=5).

Conclusions: This study found high prevalence of Pfdhfr–Pfdhps quintuple and presence of septuple mutations. However, mutations at Pfdhfr K540E and Pfdhps A581G, major predictors for IPTp-SP failure were within the recommended WHO range. Stopping IPTp-SP is recommended in areas where the Pfdhfr K540E prevalence is >95 % and Pfdhps A581G is >10 % as SP is likely to be ineffective. Nonetheless, saturation in Pfdhfr and Pfdhps haplotypes is alarming, a search for alternative antimalarial drug for IPTp in the study area is recommended.

Background

Antimalarial drugs use and vector control are the commendable tools for global malaria prevention and control respectively [1]. However, resistance of Plasmodium to antimalarial drugs and resistance of Anopheles mosquitoes to insecticides impair the progressive fight against malaria [2]. Resistance of *Plasmodium falciparum* to previous generations of medicines, i.e. chloroquine (CQ) [3] and sulfadoxine-pyrimethamine (SP) [4] influenced the replacement of CQ with SP [5], then artemisinin-based combination therapy (ACT) as the first-line treatment for uncomplicated malaria in Tanzania [6].

The changes in malaria treatment policy were supported by evidence from molecular epidemiological resistance surveillance against SP [7], which are *P. falciparum* dihydrofolate reductase (*Pfdhfr*) and dihydropteroate synthetase (*Pfdhps*) enzymes mutations [8], [9]. Mutations in the *Pfdhps* gene at codons serine 436 to alanine (S436A), alanine 437 to glycine (A437G), lysine 540 glutamic acid (K540E), alanine

581 to glycine (A581G) and alanine 613 to serine (A613S) predicts sulfadoxine resistance whereby mutations in the *Pfdhfr* gene at codons cysteine 50 to arginine (C50R), asparagine 51 to isoleucine (N51I), cysteine 59 to arginine (C59R), Serine 108 to asparagine/threonine (S108 N/T), and isoleucine 164 to leucine (I164L) confer pyrimethamine resistance [10].

Mutations in both the *Pfdhfr/Pfdhps* genes which greatly influence SP clinical treatment failures [11] resulted SP being unfit for first -line treatment of uncomplicated malaria in Tanzania [12]. However, in 2006 the drug was limited for intermittent preventive treatment during pregnancy (IPTp-SP), in areas with moderate to high malaria transmission [13]. Pregnant women regardless of the presence or absence of malaria should administer at least three curative doses of SP with an interval of at least 1 month between the two doses, starting in the second trimester of pregnancy [14], for preventing pregnancy associated malaria and improve pregnancy outcomes [13].

The rapid widespread of *Pfdhfr/Pfdhps* genes mutations and saturation of haplotypes including quintuple (CIRNI-SGEAA) and sextuple (CIRNI-SGEGA) reported in Tanzania [10] are likely to compromise effectiveness of IPTp-SP strategy. In this regard, World Health Organization (WHO) recommends stopping IPTp-SP in areas where the *Pfdhfr* K540E prevalence is >95 % and *Pfdhps* A581G is >10 % as SP is likely to be ineffective [15].

For the purpose of guiding IPTp-SP policy and inform on the current SP resistance status, routinely surveillance of SP effectiveness using molecular markers should be implemented as recommended by WHO [15]. Therefore, a study was conducted in a general population to determine the status of mutations associated with SP resistance, more than one decade since SP was limited for IPTp use in pregnant women in Tanzania.

Methods

Study design

A hospital based surveillance of molecular markers for SP resistance was conducted between April and August 2019 at Kibiti Health Center (KHC). The study was conducted to determine the current antimalarial status of SP, more than a decade since SP were limited for IPTp for pregnant women in Tanzania. Tanzania, sub-Saharan country where malaria is endemic adopted the use of SP for malaria prevention in pregnancy women for more than one decade [12].

Study area

KHC is found in Kibiti District, Pwani region. Pwani region is one of the five regions found along the coastal of Indian Ocean. Pwani region is bordered to north by Tanga region, to the east by Dar es Salaam region and the Indian Ocean, to the south by the Lindi region, and to the west by the Morogoro region. The

coastal region experiences malaria transmission throughout the year with regional prevalence ranging between 3.1 and 14.8% [16].

Study population

Patients attending clinic at KHC who presented with symptoms suggestive of malaria infection were recruited in the study. The symptoms such as fever, general body weakness and headache were confirmed by the attending physician [17]. Malaria screening in patients were done using CareStart™ malaria HRP2/pLDH (Pf/pan) Combo test (Access Bio, Ethiopia). Then positive samples by rapid tests were subjected to blood smear (BS) microscopy for confirmation. A total of 489 dried blood samples (DBS) from patients tested positive with BS microscopy were prepared.

DBS preparation and DNA extraction

Dried Blood Spots (DBS) were prepared according to MalariaGEN SpotMalaria, DBS collection protocol [18]. A sterilized patient's finger was pricked, and four blood spots from each patient were prepared; two on each paper card. The blood spots were allowed to dry and placed in the desiccant sachet for storage. DNA from the DBS was extracted using QIAamp DNA Investigator Kit for isolation of total DNA from filter papers (Qiagen, Valencia, CA, USA) and as described by Oyola *et al*.,2016 [19].

Genotyping of *Pfdhfr* and *Pfdhps* mutations

Molecular genotyping of *Pfdhfr* and *Pfdhps* gene mutations associated with SP-resistance was performed by Wellcome Sanger Institute, UK.

Briefly, targets for genotyping were identified and multiplex PCR primers were designed using a modified version of the mPrimer software [20] and in accordance to Wellcome Sanger Institute protocol (Goncalves, manuscript in preparation). Primers were designed to amplify products between 190-250bp and combined into 3 pools. A two-step protocol was used to first amplify the target regions of the parasite genome, followed by a second PCR to incorporate sequencing and multiplexing adapters. Batched samples (384) were sequenced in a single MiSeq lane combining all PCR products. Samples were de-plexed using the multiplexing adapters and individual CRAM files were aligned to a modified amplicon reference genome. Genotyping was done using bcftools as well as custom scripts to filter and translate genotypes into *Pfdhfr* and *Pfdhps* resistance haplotypes

Statistical analysis. Laboratory data on Microsoft Excel sheet (Redmond, WA) were exported directly to Statistical Package for Social Sciences version 25 (SPSS Software, Chicago Inc., USA) for data analysis. Haplotypes were expressed as frequencies and percentages.

Results

Haplotypes for *Pfdhfr* and *Pfdhps*

The *Pfdhfr* was analyzed from 445 samples, the wild type (WT) *Pfdhfr* haplotype NCSI was detected in only 1.3% (number [n] = 6) samples. Triple *Pfdhfr* **IRN**I haplotype was dominant, contributing to 84% (n = 374) of haplotypes. The total of 446 samples were studied for *Pfdhps*. The WT for *Pfdhps* was found in 6.7% (n = 30) of all samples detected. The most common mutation was the change of amino acid alanine to glycine (A437G) and lysine to glutamic acid (K540E). Double *Pfdhps* haplotype (S**GE**AA) accounted for 83% of mutations of the *Pfdhps* gene (Table 1) . The overall prevalence of K540E was 90.4% (n=396) while A581G was 1.1% (n = 5)

Table 1: *Pfdhfr* and *Pfdhps* haplotypes

Combined *Pfdhfr* and *Pfdhps* haplotypes

Overall 447 (91.4%) genotypes were detected from 489 sequenced samples. The concomitant mutations (*Pfdhfr-Pfdhps*) were present in 99.1% (n = 443) of samples. Of 447 genotypes, only 4 samples (0.9%) were WT (SAKAA-NCSI). Concomitant mutations with quintuple haplotype (S**GE**AA-**IRN**I) dominated by 71.4% whereby sextuple haplotype (**AG**K**GS**-**IRN**I) was detected in one sample (0.2%) (Table 2).

Table 2: Combined *Pfdhfr-Pfdhps* haplotypes

Discussion

This study was conducted to determine the current SP resistance status, more than a decade since SP was limited for IPTp use in pregnant women in Tanzania.

The study found high level (99.1%) concomitant mutations (*Pfdhfr-Pfdhps*) where quintuple mutation (S**GE**AA-**IRN**I) dominated by 71.4%. Additionally, one sample detected septuple mutation (**AG**K**GS**-**IRN**I) and only 0.9% contained the WT (SAKAA-NCSI) protein. These results were close to the findings from the previous study which reported high-level of *P. falciparum* SP resistance with concomitant occurrence of septuple haplotype [10]. There was a high number of quintuple (S**GE**AA-**IRN**I) and few sextuple mutations. These haplotypes have been highly associated with sub-optimal IPTp-SP effectiveness in study conducted between 2008 – 2010 in Korogwe district, Tanzania [21]. Moreover, high prevalence of the *Pfdhfr/Pfdhps* sextuple haplotype was associated with reduced birth- weight [22]–[24].

In this study, triple *Pfdhfr* **IRN**I haplotype mutation was dominant, contributing to 84% of all *Pfdhfr* haplotypes whereas double *Pfdhps* haplotype (S**GE**AA) accounted for 83% of mutations of the *Pfdhps*

haplotypes. Compared to study by Baraka *et al*.,2015 [10], *Pfdhfr* **IRN**I haplotype were predominant in all sites with significantly higher frequencies at Muheza (93.3 %) compared to Muleba (75.0 %) and Nachingwea districts (70.6 %). In this regard, there was 13.4% increase in *Pfdhfr* **IRN**I haplotype prevalence when Nanchingwea (70.6%) and Kibiti district (84%) both found along the coastal region were compared. Additionally, the previous study found that double *Pfdhps* haplotype S**GE**AA was significantly high at Muheza (27.2 %) and Muleba (20.8 %) while none (0 %) was detected at Nachingwea while the current study at Kibiti district detected 83% *Pfdhps* S**GE**AA mutations. Meaning that, from 2015 to 2019 there is an increase of 0% to 83% *Pfdhps* S**GE**AA mutation in the coastal region, respectively.

WHO malaria report of 2019 has clearly mentioned pregnant women as the most affected group and use of effective IPTp should be highly implemented to combat risks associated with pregnancy malaria [25]. On top of that, a recent study by Mikomangwa *et al*., [26] concluded that pregnant woman who was malaria positive had 11 times more risk of giving birth to low birth weight (LBW) child when compared to those who tested malaria negative.

Nevertheless, *Pfdhfr* K540E and *Pfdhps* A581G, major predictors for IPTp-SP failure, were found to have the prevalence of 90.4% for *Pfdhfr* K540E and 1.1% for *Pfdhps* A581G. This guarantee the continued use of IPTp-SP in the study area. Since, WHO recommends stoppage of SP for IPTp if *Pfdhfr* K540E prevalence >95 % and *Pfdhps* A581G >10 % as SP is likely to be ineffective [15]. However, rapid increase and saturation in *Pfdhfr* and *Pfdhps* mutations with spread of sextuples and septuple is of great concern. The effort to search for alternative drug (s) to replace SP for IPTp should be prioritized. The current study was conducted in the general population, hence limited data on clinical, birth outcomes and malaria infection status in pregnant women in relation to SP resistance genotypes.

Conclusions

This study found high prevalence of *Pfdhfr*–*Pfdhps* quintuple mutations. However, mutations at *Pfdhfr* K540E and *Pfdhps* A581G, major predictors for IPTp-SP failure were within the recommended WHO range. Stopping IPTp-SP is recommended in areas where the *Pfdhfr* K540E prevalence is >95 % and *Pfdhps* A581G is >10 % as SP is likely to be ineffective. Saturation in *Pfdhfr* and *Pfdhps* haplotypes is alarming, a search for alternative antimalarial drug for IPTp, the study area in particular is recommended.

Abbreviations

SP: sulfadoxine-pyrimethamine

WT: wild type

IPTp: intermittent preventive treatment during pregnancy

Pfdhfr: *Plasmodium falciparum* dihydrofolate reductase

Pfdhps: *Plasmodium falciparum* dihydropteroate synthetase

WHO: World Health Organization

Declarations

Ethics approval and consent to participate

Ethical approval to conduct this study was obtained from Muhimbili University of Health and Allied Sciences Ethical Review Board (Ref. DA.282/298/01A.C/) and National Institute for Medical Research (Ref. NIMR/HQ/R.8A/Vol.IX/3107). Permission to conduct the study at KHC was obtained from both Kibiti District Medical Officer and KHC Medical Officer In-charge. Written informed consents after explaining the purpose of the study were requested before enrollment.

Competing interests

The authors declare that they have no competing interests.

Availability of data

The datasets generated and/or analysed during the current study are available from the corresponding author on reasonable request.

Consent for publication

Not applicable

Funding

This study was funded by Swedish International Development Cooperation Agency (Sida), Sweden through Muhimbili University of Health and Allied Sciences. The funder did not participate in the design of the study, data collection, analysis, interpretation, and manuscript preparation.

Authors' contributions

GMB participated in conception; study design, data collection, analysis and manuscript writing, MK, WPM participated in data analysis and manuscript writing. All authors read and approved the final manuscript.

Acknowledgements

This publication uses data generated using Tanzanian samples in collaboration with MalariaGEN SpotMalaria Project (https://www.malariagen.net/projects/spotmalaria); the project is coordinated by the MalariaGEN Resource Centre with funding from Wellcome (206194, 090770). The authors would like to thank the staff of Wellcome Sanger Institute Sample Management, Genotyping, Sequencing and Informatics teams for their contribution. Furthermore, we thank Sonia Goncalves for providing the statement on the molecular typing for antimalarial resistance markers. The author (GMB) acknowledge the training on antimalarial resistance markers when he attended Global Health Fellowship 2018, Novartis Institutes for Biomedical Research, Emeryville, CA, USA. Special thanks to Prof. A. Kamuhabwa and Dr. B. Ngasala for their mentorship support.

Authors' Information

[1]Department of Pharmaceutical Microbiology, Muhimbili University of Health and Allied Sciences, P.O. Box 65013, Dar es Salaam, Tanzania.

[2]Department of Clinical Pharmacy and Pharmacology, Muhimbili University of Health and Allied Sciences, P.O. Box 65013, Dar es Salaam, Tanzania.

References

[1] E. Patouillard, J. Griffin, S. Bhatt, A. Ghani, and R. Cibulskis, "Global investment targets for malaria control and elimination between 2016 and 2030," *BMJ Glob. Heal.*, vol. 2, no. 2, p. e000176, May 2017.

[2] WHO, "Global report on antimalarial drug efficacy and drug resistance: 2000-2010," *Who*, p. 115, 2010.

[3] A. Mohammed *et al.*, "Trends in chloroquine resistance marker , Pfcrt-K76T mutation ten years after chloroquine withdrawal in Tanzania," *Malar. J.*, vol. 12, no. 1, p. 1, 2013.

[4] M. NDEJEMBI *et al.*, "Therapeutic Efficacy of Sulfadoxine-Pyrimethamine and Prevalence of Resistance Markers in Tanzania Prior To Revision of Malaria Treatment Policy: Plasmodium Falciparum Dihydrofolate Reductase and Dihydropteroate Synthase Mutations in Monitoring in Vivo Re," *Am. J. Trop. Med. Hyg.*, vol. 71, no. 6, pp. 696–702, 2018.

[5] G. M. Mubyazi and M. A. Gonzalez-Block, “Research influence on antimalarial drug policy change in Tanzania: Case study of replacing chloroquine with sulfadoxine-pyrimethamine as the first-line drug,” *Malar. J.*, vol. 4, pp. 1–13, 2005.

[6] Y. P., C. J.L., A. S., A. J., L. P.S., and M. J., “Trends in availability and prices of subsidized ACT over the first year of the AMFm: Evidence from remote regions of Tanzania,” *Malar. J.*, vol. 11, p. 299, 2012.

[7] K. Mugittu *et al.*, “Efficacy of sulfadoxine-pyrimethamine in Tanzania after two years as first-line drug for uncomplicated malaria: assessment protocol and implication for treatment policy strategies,” *Malar J*, vol. 4, 2005.

[8] D. R. Brooks, P. Wang, M. Read, W. M. Watkins, P. F. G. Sims, and J. E. Hyde, “Sequence Variation of the Hydroxymethyldihydropterin Pyrophosphokinase: Dihydropteroate Synthase Gene in Lines of the Human Malaria Parasite, Plasmodium falciparum, with Differing Resistance to Sulfadoxine,” *Eur. J. Biochem.*, vol. 224, no. 2, pp. 397–405, 1994.

[9] T. Triglia, J. G. T. Menting, C. Wilson, and A. F. Cowman, “Mutations in dihydropteroate synthase are responsible for sulfone and sulfonamide resistance in Plasmodium falciparum,” *Proc. Natl. Acad. Sci. U. S. A.*, vol. 94, no. 25, pp. 13944–13949, 1997.

[10] V. Baraka *et al.*, “High-level Plasmodium falciparum sulfadoxine-pyrimethamine resistance with the concomitant occurrence of septuple haplotype in Tanzania,” *Malar. J.*, vol. 14, no. 1, pp. 1–9, 2015.

[11] K. Mugittu *et al.*, “Therapeutic efficacy of sulfadoxine-pyrimethamine and prevalence of resistance markers in Tanzania prior to revision of malaria treatment policy: Plasmodium falciparum dihydrofolate reductase and dihydropteroate synthase mutations in monitoring in vivo re,” *Am. J. Trop. Med. Hyg.*, vol. 71, no. 6, pp. 696–702, 2004.

[12] R. A. Kavishe *et al.*, “Molecular monitoring of Plasmodium falciparum super-resistance to sulfadoxine-pyrimethamine in Tanzania,” *Malar. J.*, vol. 15, no. 1, pp. 1–8, 2016.

[13] “IT Committee: This Friday we have two items on our agenda. 1. Al Wysocki,” p. 226, 2017.

[14] S. J. Rogerson, V. Mwapasa, and S. R. Meshnick, “Malaria in pregnancy: Linking immunity and pathogenesis to prevention,” *Am. J. Trop. Med. Hyg.*, vol. 77, no. SUPPL. 6, pp. 14–22, 2007.

[15] R. Gana, F. Leke, and L. Slutsker, “WHO Evidence Review Group on Intermittent Screening and Treatment (ISTp) and ACT Treatment of Malaria in Pregnancy,” no. September, pp. 1–17, 2015.

[16] TMIS, “Tanzania Malaria Indicator Survey (TMIS) 2017,” 2017.

[17] The united republic of Tanzania standard treatment guidelines and essential medicines list, 2013.

[18] U. MalariaGEN Resource Centre members based at the Wellcome Trust Sanger Institute, “Protocol for collecting dried blood spots from malaria patients, for the purposes of sequencing Plasmodium parasites.,” *https://www.malariagen.net/network/capacity-building/methods#DBS*.

[19] S. O. Oyola *et al.*, “Whole genome sequencing of Plasmodium falciparum from dried blood spots using selective whole genome amplification,” *Malar. J.*, vol. 15, no. 1, pp. 1–12, 2016.

[20] Z. Shen *et al.*, “MPprimer: A program for reliable multiplex PCR primer design,” *BMC Bioinformatics*, vol. 11, 2010.

[21] D. T. R. Minja *et al.*, “Plasmodium falciparum mutant haplotype infection during pregnancy associated with reduced birthweight, Tanzania,” *Emerg. Infect. Dis.*, vol. 19, no. 9, pp. 1446–1454, 2013.

[22] A. M. McCollum, K. Mueller, L. Villegas, V. Udhayakumar, and A. A. Escalante, “Common origin and fixation of Plasmodium falciparum dhfr and dhps mutations associated with sulfadoxine-pyrimethamine resistance in a low-transmission area in South America,” *Antimicrob. Agents Chemother.*, vol. 51, no. 6, pp. 2085–2091, 2007.

[23] I. Naidoo and C. Roper, “Mapping ‘partially resistant’, ‘fully resistant’, and ‘super resistant’ malaria,” *Trends Parasitol.*, vol. 29, no. 10, pp. 505–515, 2013.

[24] W. E. Harrington, R. Morrison, M. Fried, and P. E. Duffy, “Intermittent Preventive Treatment in Pregnant Women Is Associated with Increased Risk of Severe Malaria in Their Offspring,” *PLoS One*, vol. 8, no. 2, pp. 2–7, 2013.

[25] World Health Organization (WHO), “World Malaria Report,” 2019.

[26] W. P. Mikomangwa, M. Oms, E. Aklillu, and A. A. R. Kamuhabwa, “Adverse birth outcomes among mothers who received intermittent preventive treatment with Sulphadoxine-Pyrimethamine in the low malaria transmission region,” *BMC Pregnancy Childbirth*, vol. 19, no. 1, pp. 1–11, 2019.

Tables

Table 1.

Gene	Haplotype	Frequency (n)	Percentage (%)
Pfdhfr (n=445)	NCSI	6	1.3
	I* **	1	0.2
	IC**N**I	30	6.7
	INI*	5	1.1
	IR **	5	1.1
	IRNI	374	84.0
	N**I** **	1	0.2
	N**R** **	1	0.2
	N**RN**I	22	4.9
Pfdhps (n=446)	SAKAA	30	6.7
	AAAA*	1	0.2
	AAKAA	6	1.3
	AGEAA	6	1.3
	AGK**GS**	1	0.2
	FGKAA	1	0.2
	GEAA*	2	0.4
	HAKAA	2	0.4
	SAAA*	1	0.2
	SA**E**AA	3	0.7
	S**E**AA*	3	0.7
	S**G*** **	2	0.4
	S**G**AA*	5	1.1
	S**GE** **	6	1.3
	S**GE**A*	1	0.2
	S**GE**AA	370	83.0
	S**GEG**A	4	0.9
	S**GK**AA	2	0.4

*Indicates missing genotype(s) during sequencing. Each letter represents an amino acid, A: alanine; C: cysteine; E: Glutamic acid; F: Phenylalanine; G: Glycine; H: Histidine; I: Isoleucine; K: Lysine; N: Asparagine; R: arginine; S: Serine. Bold underline represent amino acid changes. SAKAA and NCSI represent the wildtype (WT) amino acids for *Pfdhps* and *Pfdhfr*, respectively.

Table 2.

No. of *Pfdhps*- *Pfdhfr* mutations (haplotype where applicable)	Frequency (n)	Percentage (%)
Wild type (SAKAA-NCSI)	4	0.9
Single (1)*	0	0
Double (2)*	11	2.5
Triple (3)*	30	6.7
Quadruple (4)*	73	16.3
Quintuple (5) (S**GE**AA-**IRN**I)	319	71.4
Sextuple (6)*	9	2.0
Septuple (7) (**AG**K**GS**-**IRN**I)	1	0.2
Total	447	100

*The haplotype(s) type were not indicated if the mutation category had multiple haplotypes